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International Journal of Pharmaceutics 288 (2005) 73–80
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Camptothecin-catalyzed phospholipid hydrolysis in liposomes

Ann Mari Sætern*, Merete Skar, Åsmund Braaten, Martin Brandl

Institute of Pharmacy, University of Tromsø, Breivika, N-9037 Tromsø, Norway

Received 20 April 2004; received in revised form 1 September 2004; accepted 10 September 2004
Available online 10 November 2004

Abstract

Hydrolysis of phospholipid (PL) within camptothecin (CPT)-containing liposomes was studied systematically, after elevated lyso-phosphatidylcholine (LPC)-concentrations in pH 5, CPT-containing liposomes (22.1 ± 0.9 mol%) relative to control-liposomes (7.3 ± 0.5 mol%) occasionally had been observed after four months storage in fridge. Liposomes were prepared by dispersing freeze–dried PL/CPT mixtures in 25 mM phosphate buffered saline (PBS) of varying pH (5.0–7.8) and CPT concentrations (0, 3 and 6 mM). PL-hydrolysis was monitored by HPTLC, quantifying LPC. In an accelerated stability study (60 °C), a catalytic effect of CPT on PL-hydrolysis was observed after 40 h, but not up to 30 h of incubation. The pH profile of the hydrolysis indicated a stability optimum at pH 6.0 for the liposomes independent of CPT. The equilibrium point between the more active lactone- and the carboxylate-form of CPT was found to be pH 6.8. As a compromise, pH 6.0 was chosen, assuring > 85% CPT to be present in the lactone form. At this pH, both control- and CPT-liposomes showed only minor hydrolysis after autoclaving (121 °C, 15 min). Storage at room temperature and in fridge (2 months), as well as accelerated ageing (70 °C, 25 h), gave a significant elevation of LPC content in CPT-liposomes relative to control-liposomes. This study demonstrates a catalytic effect of CPT on PL-hydrolysis, the onset of which seems to require a certain threshold level of hydrolytic degradation.
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Keywords: Lyso-phosphatidylcholine; Stability; Phospholipids; Hydrolysis; Camptothecin; Liposomes

1. Introduction

The aim of this work was to investigate more systematically the instability of camptothecin (CPT)-containing liposomes, which was occasionally observed, and to examine whether CPT has an impact on the storage stability of liposomes in terms of hydrolysis of phosphatidylcholine (PC). The stability of phospholipids (PL) is crucial for the toxicological and therapeutic effect of liposome drug carriers. The chem-

Abbreviations: CPT, camptothecin; EPC, egg-phosphatidylcholine; HPTLC, high-performance thin layer chromatography; LPC, lyso-phosphatidylcholine; MLV, multilamellar vesicle; PC, phosphatidylcholine; PL, phospholipid; VPG, vesicular phospholipid gel

* Corresponding author. Tel.: +47 7764 6640; fax: +47 7764 6151.
E-mail address: annmari@farmasi.uit.no (A.M. Sætern).

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doi:10.1016/j.ijpharm.2004.09.010

ical degradation of PC mainly happens by two processes: oxidation and hydrolysis. Oxidation generally does not limit the shelf-life as long as proper measures has been taken, i.e. use of antioxidants, filling with nitrogen and storage in the dark (Crommelin et al., 1994, Hernandezcaselles et al., 1990). Hydrolytic decomposition of PL finally leads to free fatty acids and glycerol–phosphoric acid esters. Since the first step of PC-hydrolysis appears to be rate limiting, the intermediate product, lyso-phosphatidylcholine (LPC), is often taken as a measure for determination of hydrolysis rate of PC in liposome dispersions (Moog et al., 2000; Hernandezcaselles et al., 1990). Various measures have been suggested to minimize hydrolytic degradation of PL in aqueous liposome dispersions, such as selecting the optimal pH, buffer composition, low storage temperature, and freeze–drying of liposomes (Grit et al., 1989; Grit and Crommelin, 1993). Hydrolysis is known to both effect the physical stability and safety of liposomes (Grit and Crommelin, 1992, 1993; Lutz et al., 1995).

CPT is a plant alkaloid with potent antitumor activity. It has a pentacyclic structure with a dynamic pH-dependent equilibrium between the closed ring lactone and open-ring carboxylic acid form (Fig. 1). At neutral and alkaline pH, the less active carboxylate form is dominant. The active and more hydrophobic lactone form is favored by acidic pH and is stabilized by hydrophobic environments like phospholipid bilayers present in cell membranes, erythrocytes and liposomes (Burke et al., 1993). Liposomes also tend to protect CPT from binding to human serum albumin, which has higher binding affinity towards the carboxylate than the lactone form and drives the equilibrium towards the inactive form of the drug in human plasma (Burke et al., 1995). Poor in vivo stability and low water solubility makes liposomes an attractive formulation approach for CPT delivery, as liposomes are expected to stabilize and solubilize the drug so that it is present in therapeutically relevant concentrations and in the active hydrophobic form of the drug (Burke et al., 1993; Daoud et al., 1995; Cortesi et al., 1997; Lundberg, 1998; Lynam et al., 1999).

In this study we have focused on the effect of CPT on the stability of a CPT-liposome formulation against hydrolysis.

2. Materials and methods

2.1. Materials

(*S*)-(+)-Camptothecin was obtained from Boehringer Ingelheim Pharma, Germany. The phospholipid (PL) used, Lipoid E-80 (80–85% egg-phosphatidylcholine (EPC)), was delivered by Lipoid GmbH, Germany. L-α-Lyso-phosphatidylcholine (LPC) was purchased from Sigma–Aldrich, Germany. Organic solvents were obtained from Merck, Germany, and were of gradient grade quality for liquid chromatography unless stated otherwise. Triethylamine (for analysis), acetic acid (glacial 100%), di-sodium hydrogen phosphate dihydrate (extra pure), potassium di-hydrogen phosphate (pro analysi) and sodium hydroxide pellets (extra pure) were purchased from Merck, Germany. Water was freshly distilled and buffers filtrated through 0.22-μm pore-size filters prior to use. HPTLC plates with silicagel 60, 100 mm × 200 mm, were obtained from Merck, Germany.

2.2. Liposome preparation

In order to prepare a homogeneous blend of PL (Lipoid E-80) and CPT, solutions of CPT and PL in DMSO and methanol, respectively, were mixed. The PL–CPT solution was shock frozen in liquid nitrogen and freeze–dried for 65 h at temperatures from −40 to 45 °C and pressures decreasing from 800 to 0.008 mbar.

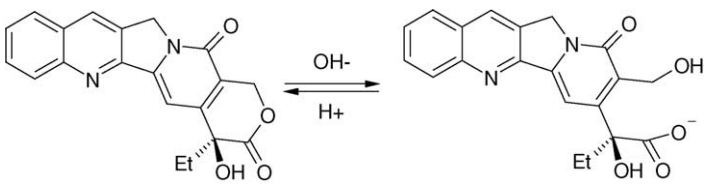

Fig. 1. The camptothecin lactone–carboxylate equilibrium.

The freeze–dried PL–CPT cake was stored at −80 °C. Control samples contained lipid (PL) only.

The freeze–dried PL–CPT cakes were brought to room temperature and 25 mM PBS added. The total batch size was 40 g for the vesicular phospholipid gel (VPG)-liposomes, containing 40% (w/w) PL, and 2.0 ml for the multilamellar vesicles (MLV)-liposomes containing 10% (w/w) PL. The MLV-liposomes were prepared by dispersing on a MS2 Minishaker (IKA® company group). The 40% VPGs were prepared by swelling for 60 min followed by homogenization in a high-pressure homogenizer (APV Micron LAB 40, APV homogenizer, Germany), set at 70 MPa for 10 cycles. Where indicated, CPT-liposome and control-liposome (VPGs) were transferred to 10-ml injection vials, sealed and autoclaved for 15 min at 121 °C in a steam sterilizer (CertoClave Type CV-EL 10L/12L, CertoClav Sterilizer GmbH, Traun, Austria).

2.3. Stability study—ageing conditions

The CPT-liposomes and control-liposomes (MLVs) were prepared in PBS (pH 5.0, 5.4, 5.8, 6.2, 6.6, 7.0, 7.4 or 7.8) and the hydrolysis studied upon incubation. For stability testing, samples were stored at room temperature (20–22 °C) or in fridge (4–8 °C), protected from light and in well-sealed containers. Accelerated ageing was performed in a shaking water bath (GFL 1086, GFL GmbH, Germany) at 60 or 70 °C for up to 40 h at 70 rpm.

2.4. High-performance thin layer chromatography (HPTLC) analysis of LPC

Liposome samples were dissolved and diluted in methanol prior to HPTLC analysis. Lipid concentrations in the dispersions were 10 and 40% (w/w), respectively. The volume ratio between the liposome preparation and methanol solvent was varied to reach a final PL concentration of between 1 and 4 mg/ml or LPC concentrations within the range of the standard curve (25.6–255.5 μg/ml) using sample volumes of between 10 and 25 ml.

HPTLC plates, silicagel 60, 100 mm × 200 mm were pre-run in 60 ml mobile phase; ($CHCl_3$/MeOH/triethylamine/water (30:35:34:8, v/v)) (Kötting et al., 1992). Every formulation was analyzed in triplicate, and both samples and standards were applied in duplicate to every HPTLC plate with a CAMAG Linomat III applicator (Camag, Muttenz, Switzerland). Application parameters were as follows; volume 10 μl, dosage speed 5 s/μl, 20 bands, band length 5 mm, space between bands 3.7 mm, space from lower edge of the plate 15 mm.

After evaporation of the sample solvents, the plates were developed in a closed flat bottom chamber (20 cm × 10 cm) containing 70 ml mobile phase. The development was stopped when the solvent had migrated approximately 65 mm. The plates were dried at room temperature for 10 min before further drying on a CAMAG TLC Plate Heater III at 130 °C for 10 min.

The HPTLC plates were post-chromatographic derivatizated by dipping 3 × 5 s into a 10% $CuSO_4$ solution, acidified with 80 ml/l 85% phosphoric acid (Kötting et al., 1992). HPTLC plates were dried in an oven (Memmert UM100, Memmert GmbH + Co, Schwabach, Germany) at 150 °C for 30 min and thereafter on a heating plate (CAMAG TLC heater III, CAMAG, Muttenz, Switzerland) at 200 °C under a stream of air for 20–45 min prior to scanning. LPC was quantified densitometrically at 530 nm (CAMAG TLC scanner 3, Muttenz, Switzeland), using a scanning speed of 5 mm/s, a slit dimension of 3.00 mm × 0.45 mm (micro) and a mercury (Hg) lamp. A typical HPTLC plate is shown in Fig. 2. Calibration was performed by applying standard solutions of LPC in methanol in concentrations of 25.6, 51.1, 153.3 and 255.5 μg/ml, validated (Shah et al., 1992) to be within the quantification limit of the method, which was 21.33 μg/ml (corresponding to 417 pmol final amount). LPC had a R_f value of approximately 0.16 as detected visually and densiometrically by scanning (Fig. 2), whereas CPT had a R_f value of approximately 0.76, and thus CPT was judged not to interfere with the quantification of LPC in the chromatogram. The CPT spots were visualized on the HPTLC plate in a Camag UV-cabinet (Camag, Muttenz, Switzerland) at 254 and 366 nm.

2.5. High-performance liquid chromatography (HPLC) quantification of CPT

The HPLC method originally described by Warner and Burke (1997) was used with some modifications: A mobile phase gradient from 25 to 35% acetonitrile during 10 min in 1% (v/v) triethylamine buffer pH 5.5 (by using acetic acid). Flow rate was 1 ml/min. A Waters

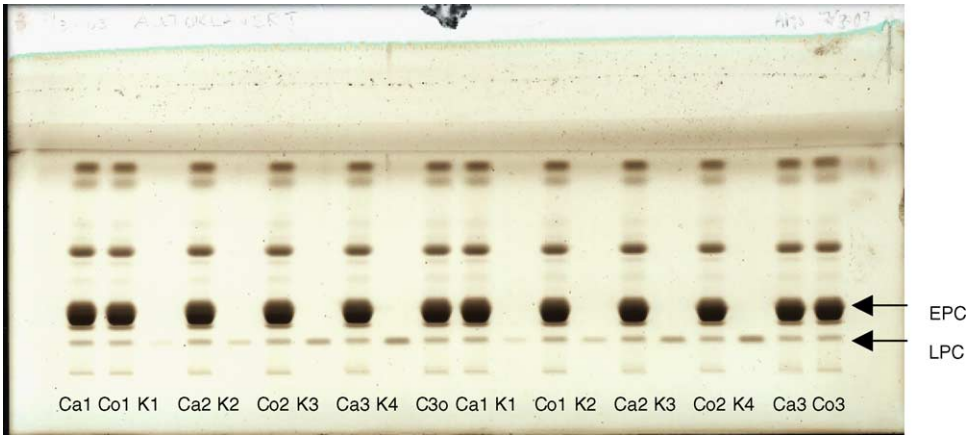

Fig. 2. A typical HPTLC plate with CPT-liposome- (Ca) and control-liposome (Co) samples and calibration solutions (K).

HPLC system was used, equipped with a 474 Scanning Fluorescence detector, a 2695 separation module, and a Symmetry® C_{18}-column, 3.9 mm × 150 mm (Waters® Milford, Massachusetts). Wavelengths: excitation $\lambda = 360$ nm and emission $\lambda = 440$. Injection volumes were either 10 or 20 μl. CPT-lactone and -carboxylate were quantified from standard curves. Standard samples were made from a 3.33 mg/ml CPT-stock-solution in DMSO. Nine millimoles of phosphate buffer (PB), pH 10.5 and pH 3.0, were used for the carboxylate- and the lactone-standards, respectively. Every standard solution was prepared in triplicate and injected twice into the HPLC.

2.6. Effect of pH on the camptothecin lactone–carboxylate equilibrium

A 50 μg/ml stock solution of CPT in DMSO was prepared and 25 μl (0.5%, w/w) thereof diluted to 5 ml in 25 mM phosphate buffer (PB) with pH 3.0, 5.5, 6.0, 6.5, 7.0, 7.5 or 11.0, to a final CPT concentration of 250 ng/ml (0.72 μM). Samples were incubated on a shaking water bath for 5 days at 25 ± 0.5 °C to assure equilibrium, and thereafter analyzed by HPLC.

3. Results

Initially in this study, unexpectedly high LPC concentration was observed after 4 months storage in fridge in CPT-liposomes (3 mg/g CPT) compared to control-liposomes without CPT. The liposomes contained PL (Lipoid E-80) and were dispersed in 25 mM PBS, pH 5.0. The acidic pH was considered necessary to assure that all CPT was in its active lactone form. The LPC content of CPT-liposomes compared to control-liposomes is displayed in Fig. 3.

3.1. Influence of camptothecin on phospholipid hydrolysis

In order to study the influence of CPT on liposome hydrolysis in more detail, 10% (w/w) liposome dispersions, pH 5.0, containing 0, 3.0 and 6.0 mM CPT were prepared and incubated at 60 °C in a shaking water bath for accelerated ageing. Samples were withdrawn and LPC content quantified after 10, 20, 30 and 40 h. At early time points, all formulations were found to have relatively similar hydrolysis rate, i.e. no significant difference in LPC content was found between CPT-containing and CPT-free liposomes (Fig. 4). After 10, 20 and 30 h of incubation, the LPC content in all samples had increased from 2.8% to approximately 3.5 and 4.5%, respectively. In contrast, the LPC content at 40 h had increased to 5.7 ± 0.1%, 6.4 ± 0.01% and 7.1 ± 0.2%, for the liposomes containing 0, 3 and 6 mM CPT, respectively. At this stage of hydrolysis, a catalytic effect of CPT seemed to appear. However, this effect was not very clear, and further evidence therefore was needed, as reported later on. It still remained unclear why this effect was not seen at earlier time points.

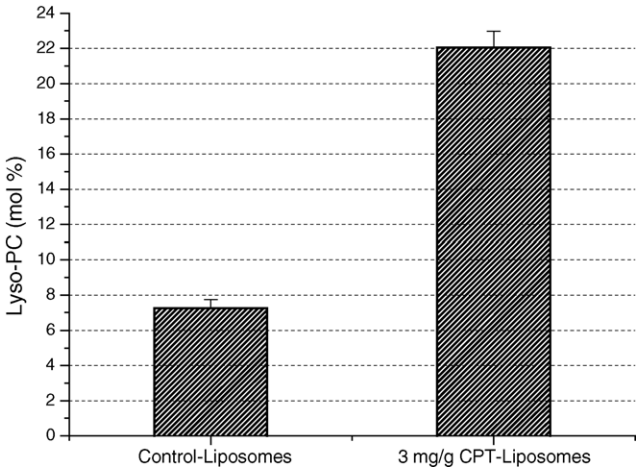

Fig. 3. Content of lyso-phosphatidylcholine in autoclaved CPT- and control-liposomes after 4 months of storage in the fridge, containing 0 and 3 mg/g CPT, respectively ($n = 2$).

3.2. Influence of pH on phospholipid hydrolysis

Liposomes with (3.0 mM) and without CPT was prepared in 25 mM PBS of varying pH and incubated for 24 h at 70 °C. The LPC contents obtained are displayed in Fig. 5. The analysis of empty liposomes resulted in a similar pH-profile (data not shown). With respect to the stability of the formulations, a pH around 6.0 appeared more favorable.

3.3. The lactone–carboxylate equilibrium of camptothecin

In order to decide how much of the CPT is present at a pH closer to 6.0, CPT was dissolved in PBS of varying pH and both the lactone and the carboxylate form were quantified by HPLC. At pH 6.0, 84.7 ± 0.1% CPT was present in its active lactone configuration (Fig. 6).

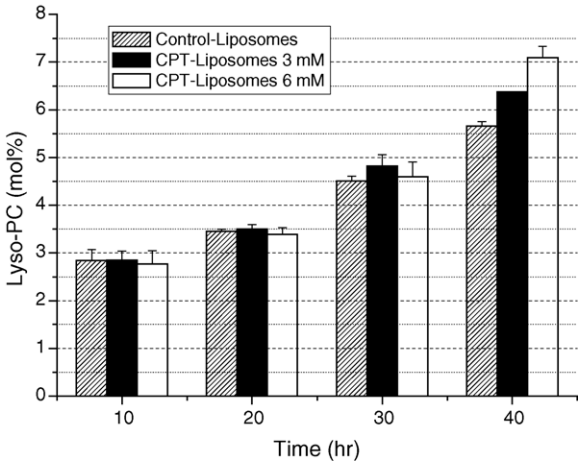

Fig. 4. Concentration of lyso-phosphatidylcholine in 10% (w/w) liposome dispersions with three different CPT concentration levels after 10, 20, 30 and 40 h incubation at 60 °C ($n = 3$).

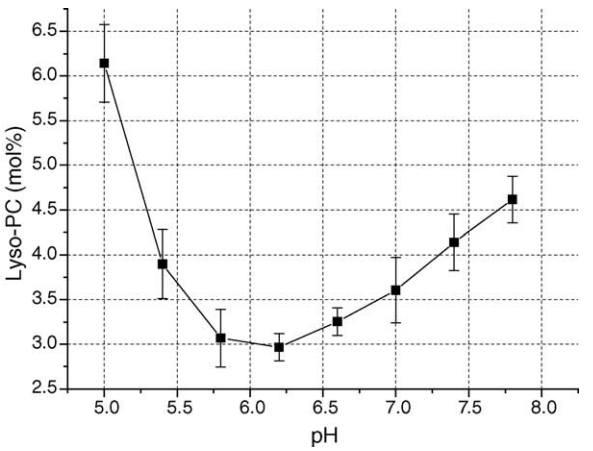

Fig. 5. pH-profile of the lyso-phosphatidylcholine content in 10% (w/w) liposome dispersions containing 3 mM camptothecin after incubation at 70 °C for 24 h ($n = 3$).

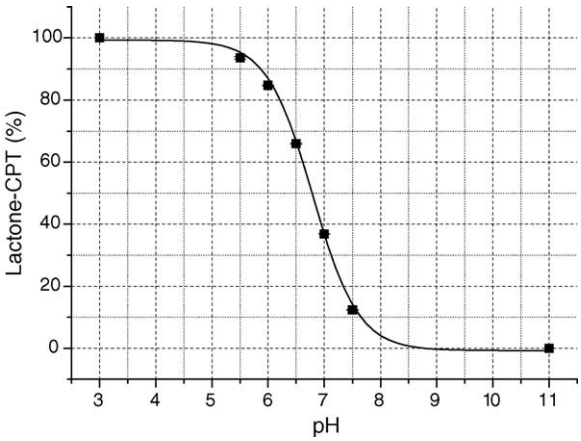

Fig. 6. The lactone–carboxylate hydrolysis equilibrium of camptothecin in PBS as function of pH ($n = 3$).

3.4. Effect of camptothecin on hydrolysis of liposomes at pH 6.0: autoclaving, accelerated ageing and storage stability

Autoclaving (121 °C and 15 min) gave a slight increase in LPC content both for the CPT-containing and the CPT-free liposomes, and no significant difference between these two formulations was observed (Fig. 7).

Liposome formulations with and without CPT (3 mM) at pH 6 were prepared, autoclaved and used both for an accelerated stability study and storage stability study in fridge (4–8 °C) and at room temperature over a period of 2 months.

In the accelerated stability study (70 °C for 25 h) an initially moderate and later on accelerating increase in LPC content was observed for both formulations, and to a higher extent in CPT-containing liposomes (Fig. 8). The LPC content was also observed to increase faster in the CPT-containing liposomes than the control during storage in fridge and at room temperature (Table 1). Hydrolysis stability was poorer at room temperature than in fridge. The observed difference in hydrolysis stability between the two formulations in both the accelerated stability study (Fig. 8) and storage stability study (Table 1) was confirmed by calculation; p-values <0.05 were attained by the student's t-test showing a significantly higher increase in LPC-concentrations for the CPT-liposome formulations as compared to the control-liposomes.

4. Discussion

The increase in LPC content observed in preliminary studies led to the assumption that there was a significant elevation of hydrolytic degradation of PL in the CPT-containing liposomes, i.e. that CPT has a catalytic effect on PL-hydrolysis. This effect could not

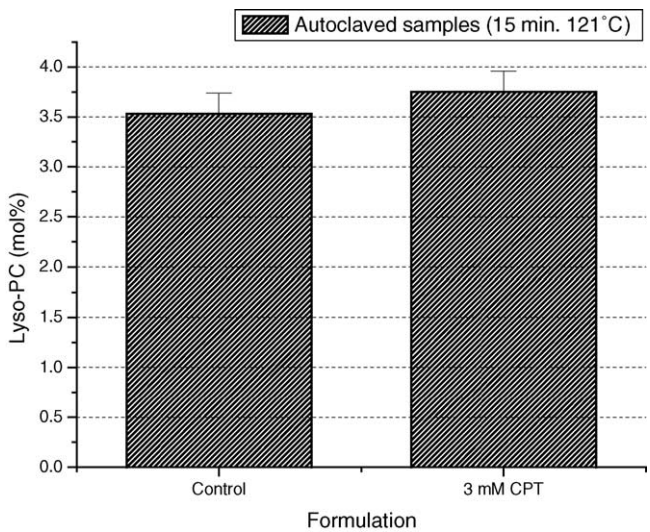

Fig. 7. Lyso-phosphatidylcholine content of autoclaved liposomes with and without camptothecin ($n = 3$).

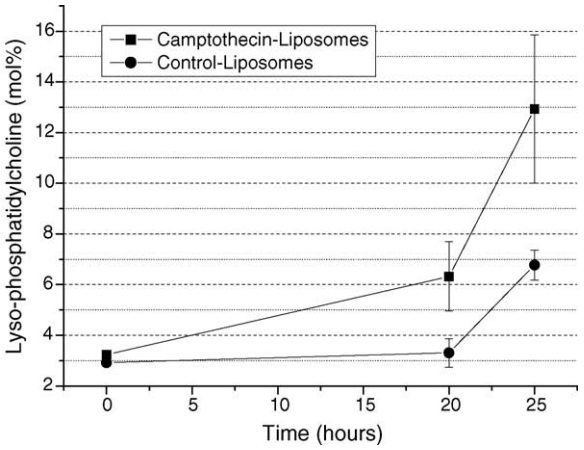

Fig. 8. Content of lyso-phosphatidylcholine in control- and CPT-liposomes after incubation at 70 °C for 20 and 25 h ($n = 3$).

be confirmed in accelerated ageing studies at 60 °C, at least not at early time points. Only after 40 h incubation were increasing amounts of LPC found with increasing CPT content of the liposomes. A possible explanation for this might be that the catalytic effect of CPT only occurs after a threshold LPC-concentration is reached in liposomes. The pH-profile of PL-hydrolysis showed that hydrolytic degradation of liposomal PLs both in presence and absence (data not shown) of CPT is at its minimum around pH 5.8–6.6, which is comparable to previous published pH-hydrolysis profiles of liposomes (Grit et al., 1989; Grit and Crommelin, 1993), where a stability optimum at pH 6.5 was observed. The pH-dependant equilibrium curve of CPT showed that the active lactone form is present at 85% in PBS at pH 6.0. According to earlier published data this equilibrium is expected to be even more on the neutral lactone side when CPT is incorporated into liposomes, as the neutral lactone-CPT is described to have a 2.5-fold higher association constant compared to the carboxylate-CPT (Burke et al., 1993). Unfortunately, the CPT-lactone/carboxylate equilibrium in liposomes is not easily measurable by HPLC due to rapid re-equilibration during sample preparation (Burke et al., 1992). It was concluded that a pH of 6.0 would represent an optimum pH in terms of an acceptable lactone–CPT concentration and minimum hydrolytic degradation of PL in the liposomes. For all further experiments, PBS of pH 6.0 therefore was used.

The process of autoclaving is of first choice to obtain a sterile product for parenteral application (Lutz et al., 1995; Zuidam et al., 1993). The effect of autoclaving on the LPC content of liposomes (pH 6.0) with and without CPT was studied, and a moderate increase in LPC content was observed irrespective whether CPT was present or not. This appears to confirm that there is no catalytic effect of CPT on PL-hydrolysis until a certain LPC level is reached. Both the accelerated ageing study and the storage stability studies indicate, however, that hydrolytic PC degradation is enhanced in the presence of CPT, and this catalytic effect of CPT appears to increase with the progress of PL-hydrolysis.

Studies on ageing of liposomes and the effect of chemical degradation on physiological stability is earlier described in literature (Grit and Crommelin, 1992; Zuidam et al., 1995). When hydrolysis exceeds 15%, permeability of the liposome bilayer increases (Grit and Crommelin, 1992). In vivo toxicity is also found when spiking liposomes with LPC at concentrations higher than 15% (Lutz et al., 1995). Further optimalization of the formulation, together with careful processing and handling, is therefore mandatory to obtain a CPT-liposome formulation with acceptable storage stability.

In conclusion, CPT has an impact on the hydrolytic PL-degradation of liposomes above a certain level of stress or lyso-lipid content. Even at the stability optimum at pH 6.0, the storage stability of CPT-containing liposomes is inadequate for a marketable pharmaceutical product.

Table 1
Lyso-phosphatidylcholine content (mol%) after autoclaving and storage at room temperature or in fridge for 0, 1 and 2 months ($n = 3$)

Time (T) (months)	Room temperature		Fridge	
	CPT-liposomes (%)	Control-liposomes (%)	CPT-liposomes (%)	Control-liposomes (%)
0	3.2 ± 0.2	2.9 ± 0.1	3.2 ± 0.2	2.9 ± 0.1
1	6.5 ± 1.4	5.0 ± 0.9	4.2 ± 0.4	3.7 ± 0.3
2	10.5 ± 0.7	6.5 ± 0.4	6.8 ± 0.9	4.9 ± 0.9

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